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G3BP1 restricts HIV-1 replication in macrophages and T-cells by sequestering viral RNA

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ABSTRACT

HIV-1 exploits the cellular machinery for replication and therefore several interactions with cellular factors take place, some of which are yet unknown. We identified GTPase-activating protein-(SH3 domain)-binding protein 1 (G3BP1) as a cellular factor that restricts HIV-1, by analyzing transcriptome profiles of *in vitro*-cytokine-activated macrophages that are non-permissive to HIV-1 replication. Silencing of G3BP1 by RNA interference resulted in increased HIV-1 replication in primary T-cells and macrophages, but did not affect replication of other retroviruses. G3BP1 specifically interacted with HIV-1 RNA in the cytoplasm, suggesting that it sequesters viral transcripts, thus preventing translation or packaging. G3BP1 was highly expressed in resting naïve or memory T-cells from healthy donors and HIV-1 infected patients, but significantly lower in IL-2-activated T-cells. These results strongly suggest that G3BP1 captures HIV-1 RNA transcripts and thereby restricts mRNA translation, viral protein production and virus particle formation.

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Introduction

Antiretroviral therapy has dramatically improved the clinical outcome of HIV-1 infection. However, total eradication of HIV-1 cannot be entirely achieved, due to the persistence of viral reservoirs that allows for continuous residual viral replication and dissemination of the infection, while avoiding triggering of immune responses. Indeed, plasma viral load in HIV-1 infected patients can very rapidly rebound after stopping therapy (Chun et al., 1999; Harrigan et al., 1999; Palmer et al., 2008). Macrophages become highly susceptible to HIV-1 infection after they differentiate from monocytic precursors (Rich et al., 1992; Schuitemaker et al., 1992; Sonza et al., 1996). HIV-1 infected macrophages found in the tissues of HIV-1 infected patients (Koenig et al., 1986) allow for residual viral replication at sites such as the gut-associated lymphoid tissue and the brain, even during antiretroviral therapy (Chun et al., 2008; Eisele and Siliciano, 2012; Lambotte et al., 2005). Thus, macrophages play a crucial role in the maintenance and dissemination of the infection. Their importance is further underscored by studies showing that they are highly resistant to the cytopathic effects of viral replication, and are able to render resting T-cells permissive for HIV-1 infection (Ancuta et al., 2006; Swingler et al., 2003). HIV-1 can only replicate in activated proliferating T-cells, where the proviral genome is integrated into the host genome. *In vivo,* these cells can go into a resting memory state, and become latent viral reservoirs (Finzi et al., 1997). These cells persist during long term therapy and are able to efficiently reinitiate virus production upon stopping therapy (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997).

The phenotype and function of macrophages are greatly determined by their activation by cytokines while differentiating in the tissues. We and others have previously shown that cytokine-mediated activation of macrophages results in inhibition of HIV-1 replication at different stages of the replication cycle: in IL-4 activated macrophages, HIV-1 is inhibited at the level of reverse transcription, whereas IFNγ+TNFα and IL-10 stimulation leads to restriction of later stages in the replication cycle (Cassetta et al., 2013; Cassol et al., 2009; Cobos Jiménez et al., 2012). Several new cellular factors have been identified to restrict early steps of HIV-1 replication upon type 1 interferon stimulation of

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macrophages, such as MX2 (Goujonet al., 2013), IFI16 (Jakobsen et al., 2013) and SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011; Lahouassaet al., 2012), or MCPIP1 in activated CD4$^+$ T cells (Liu et al., 2013). There is however a lack of knowledge regarding cellular factors that affect post-integration steps and thereby allow infected cells that harbor an integrated provirus to persist as viral reservoirs and remain undetected by the immune system.

Therefore, we aimed to identify cellular factors that could restrict HIV-1 replication in primary cells. For this purpose, we compared gene transcriptome profiles of permissive macrophages with those of non-permissive/ IFNγ+TNFα activated

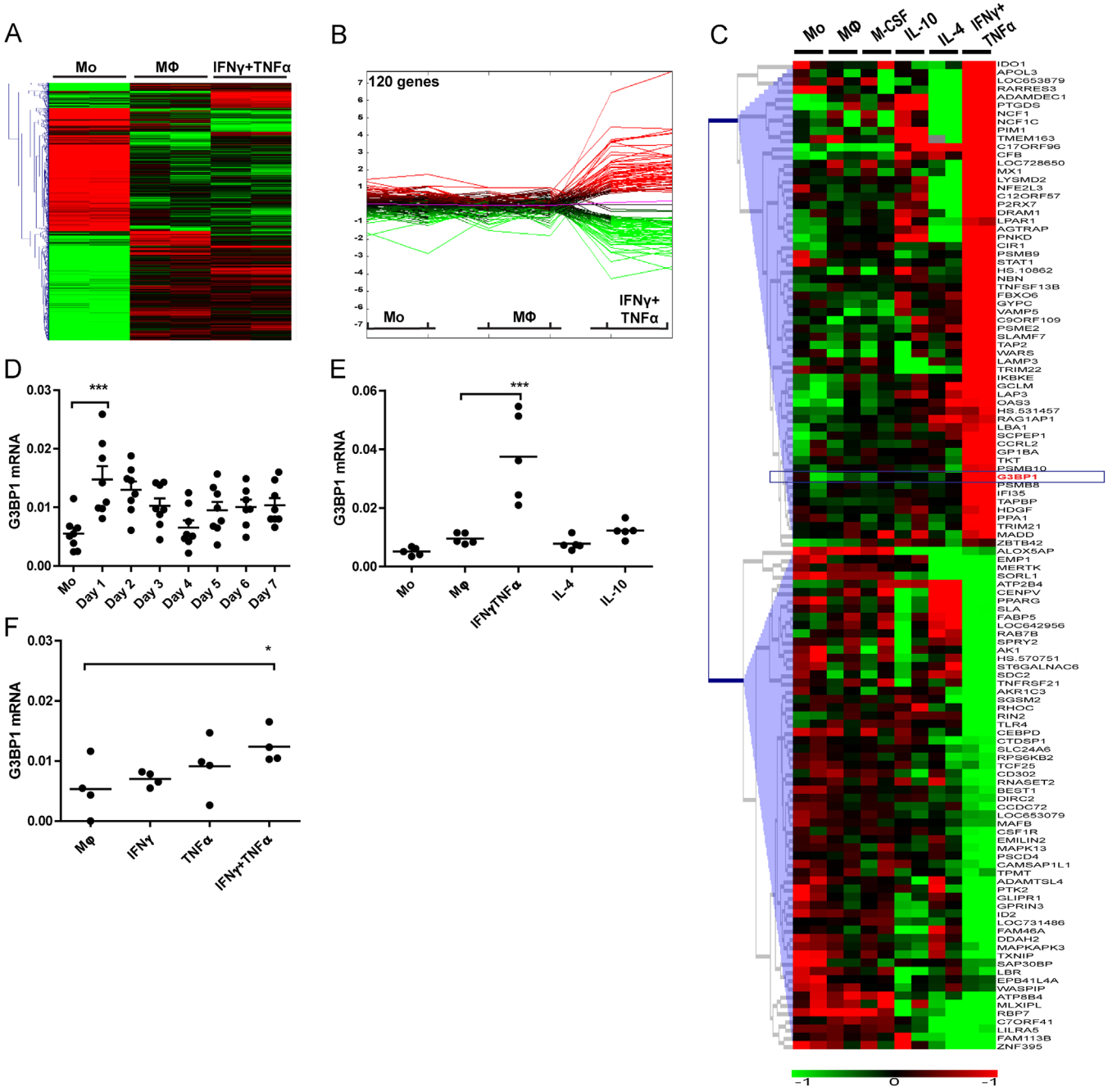

Fig. 1. IFNγ+TNFα stimulation of macrophages induces G3BP1 expression. (A) Hierarchical clustering of genes significantly induced during maturation of monocytes (Mo) into macrophages (MΦ) for 5 days and stimulation with IFNγ+TNFα (50 U/ml and 12.5 ng/ml) for 5 days, using average median-centered values from 2 donors; (B) Genes that were regulated only by stimulation with IFNγ+TNFα were selected by a Pavlidis Template Matching (PMT) analysis, using median-centered values; (C) Hierarchical clustering of expression of the PMT selected genes in monocytes (Mo), unstimulated macrophages (MΦ), or cells stimulated for 5 days with M-CSF (50 ng/ml), IL-4 (50 ng/ml) or IL-10 (50 ng/ml), using median centered values. The location of G3BP1 is indicated on the right side; (D) Expression of G3BP1 relative to a housekeeping gene, in monocytes and macrophages cultured for 1 to 7 days without cytokine stimulation, and isolated from 5 donors; (E) Expression of G3BP1 relative to a housekeeping gene, in monocytes, and unstimulated macrophages (MΦ) or stimulated with IFNγ+TNFα (50 U/ml and 12.5 ng/ml), IL-4 (50 ng/ml) and IL10 (50 nl/ml) for 5 days. (F) Expression of G3BP1 relative to a housekeeping gene, in unstimulated macrophages (MΦ) or stimulated with IFNγ (50 U/ml), TNFα (12.5 ng/ml) or IFNγ+TNFα (50 U/ml and 12.5 ng/ml). Significant differences in the expression levels are indicated by asterisks (One-way ANOVA and Bonferroni's Multiple Comparison Test, $p < 0.05$*, $p < 0.01$**, $p < 0.001$***).

macrophages. HIV-1 is restricted after integration in these activated macrophages (Cobos Jiménez et al., 2012; Cassol et al., 2009), and therefore with this strategy we could identify novel restriction factors that act at late stages of the viral cycle. We have identified a cellular factor that restricts HIV-1 at post-integration steps by sequestering viral RNA transcripts, and possibly altering translation and packaging. Identification of cellular factors that affect post-integration steps contributes to our understanding of HIV-1 replication and persistence in primary cells, and provides tools to design novel therapeutic alternatives.

Results

G3BP1 is expressed in non-permissive macrophages

To identify novel HIV-1 restriction factors that block replication after proviral integration, we compared genome-wide transcriptional profiles of human macrophages with those of IFNγ+TNFα-stimulated macrophages (Fig. 1A), where HIV-1 infection is restricted at a post-integration level (Cobos Jiménez et al., 2012). We identified 120 genes that were differentially regulated in IFNγ+TNFα stimulated macrophages, but not during differentiation of monocytes into macrophages (Fig. 1B). Expression of most of the 120 selected genes was exclusively regulated by IFNγ+TNFα stimulation, and changes in expression were neither observed in IL-4, IL-10 nor M-CSF-stimulated macrophages (Fig. 1C). From the initial selection, only 4 genes were previously identified in siRNA/shRNA genomic screens as host factors required for HIV-1 replication (Brass et al., 2008; Konig et al., 2008; Yeung et al., 2009; Zhou et al., 2008). Another 19 genes either possessed antiviral characteristics or could be involved in viral infections according to the Ingenuity Knowledge Database (Table 1). One of these genes was MX1, recently reported to be an IFN-α induced gene, capable of inhibiting influenza virus but not HIV-1 replication (Goujon et al., 2013). These data confirm that our selection strategy is suitable to identify novel antiviral genes. Notably, one of the genes that was strongly upregulated in IFNγ+TNFα-activated macrophages was G3BP1, which has been shown to be involved in infection by viruses such as Hepatitis C Virus, Dengue virus and members of the Arenavirus genus (Reineke and Lloyd, 2013).

To confirm the results from the transcriptome analyses, we measured G3BP1 levels in both monocytes and macrophages from additional donors. During maturation of monocytes into macrophages, G3BP1 levels increased on the first day of culture, but decreased progressively until they reach similar levels as those found in monocytes, after day 5 of differentiation (Fig. 1D). G3BP1 mRNA levels increased strongly upon stimulation of macrophages with IFNγ+TNFα, but not with other cytokines (Fig. 1E), IFNγ alone or TNFα alone (Fig. 1F).

G3BP1 restricts HIV-1 replication

To investigate the effect of G3BP1 on HIV-1 infection, we silenced G3BP1 expression in HEK293T cells with shRNA (Fig. 2A) and measured replication of a VSV-g-pseudotyped HIV-1-luciferase reporter virus (Fig. 2B). Notably, upon silencing of G3BP1, HIV-1 replication was significantly increased in a dose dependent manner (Fig. 2B). Additionally, silencing of G3BP1 also led to a dose dependent increase of luciferase expression from a HIV-1 LTR reporter construct (Fig. 2C), We have previously shown that in primary macrophages stimulated with IFNγ+TNFα, where G3BP1 is highly expressed, there is no inhibition of viral entry or reverse transcription (Cobos Jiménez et al., 2012). Here we observed that silencing of G3BP1 did not affect HIV-1 proviral DNA levels (Fig. 2D) but did result in increased levels of Pol mRNA copies (Fig. 2E), higher production of virus particles (Fig. 2F) and viral protein production (Fig. 2G). Together these results indicate that G3BP1 regulates HIV-1 replication at a post-transcriptional step. To explore whether G3BP1 could also affect replication of other retroviruses, we silenced G3BP1 in 293T cells and inoculated these cells with VSV-g-pseudotyped SIVmac and MLV-luciferase reporter

Table 1
Genes with antiviral characteristics, expressed in macrophages stimulated with IFNγ and TNFα.

*Type*Symbol	Entrez gene name	Fold change IFNγ+TNFα vs Mφ	Location
Enzyme			
G3BP1	GTPase activating protein (SH3 domain) binding protein 1	3.572	Nucleus
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	2.499	Nucleus
NCF1	Neutrophil cytosolic factor 1	18.016	Cytoplasm
RARRES3	Retinoic acid receptor responder (tazarotene induced) 3	25.25	Cytoplasm
RHOC	ras homolog family member C	−2.736	Plasma Membrane
TKT	Transketolase	2.361	Cytoplasm
TRIM21	Tripartite motif containing 21	2.159	Nucleus
G-protein coupled receptor			
AGTRAP	Angiotensin II receptor-associated protein	2.969	Plasma Membrane
Kinase			
IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	3.655	Cytoplasm
MERTK	C-mer proto-oncogene tyrosine kinase	−11.002	Plasma Membrane
Ligand-dependent nuclear receptor			
PPARG	Peroxisome proliferator-activated receptor gamma	−3.113	Nucleus
Peptidase			
PSMB9	Proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	6.642	Cytoplasm
Transcription regulator			
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	−3.118	Nucleus
STAT1	Signal transducer and activator of transcription 1, 91 kDa	7.482	Nucleus
TRIM22	Tripartite motif containing 22	2.428	Cytoplasm
Transmembrane receptor			
TLR4	Toll-like receptor 4	−2.746	Plasma Membrane
Other			
IFI35	Interferon-induced protein 35	2.846	Nucleus
SDC2	Syndecan 2	−7.238	Plasma Membrane
TXNIP	Thioredoxin interacting protein	−3.012	Cytoplasm

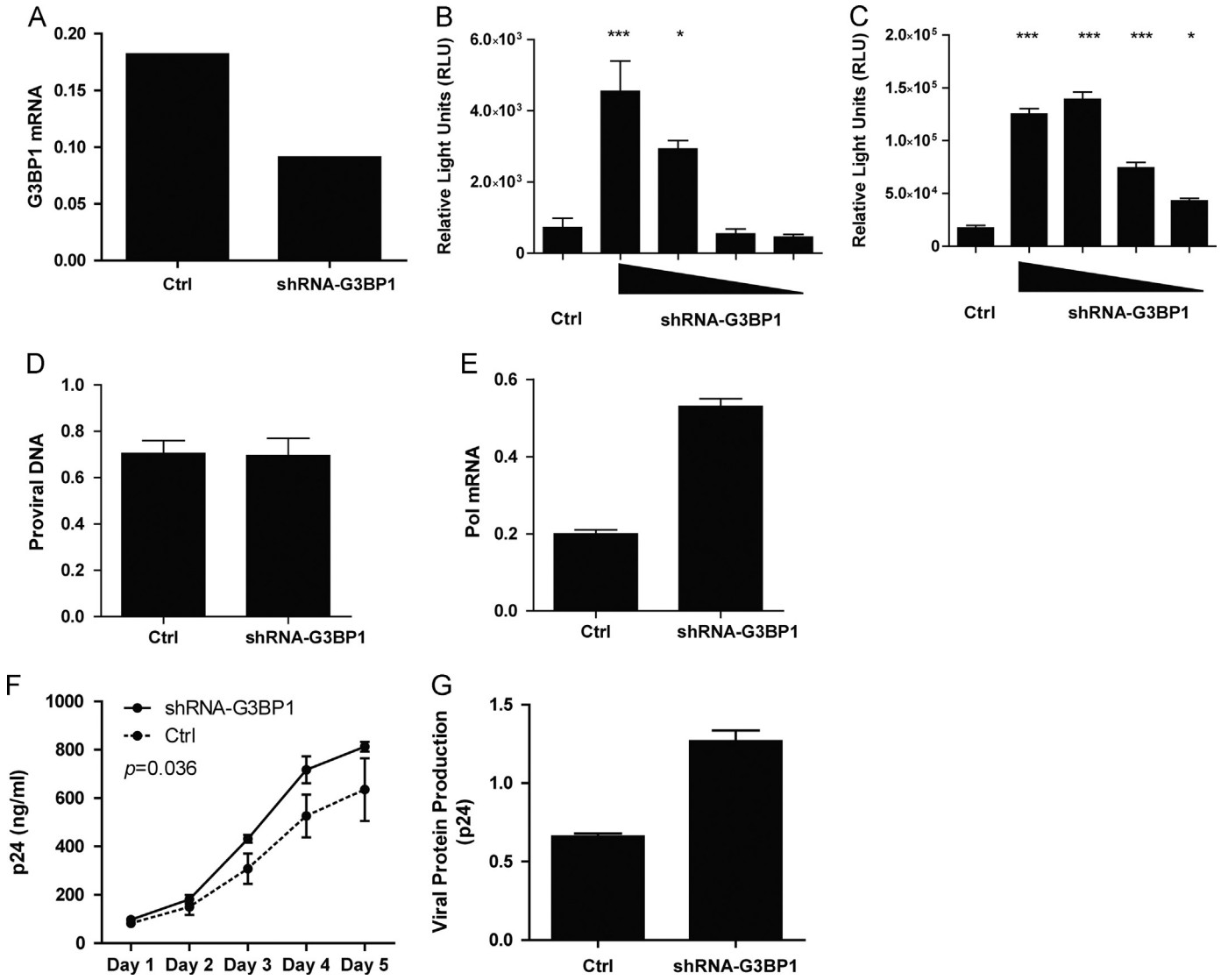

Fig. 2. G3BP1 restricts HIV-1 replication at a post-integration level. (A) 293T cells were transfected with 50 ng of shRNA-3 targeting G3BP1or shRNA control and subsequently inoculated with single round-luciferase reporter NL4-3 Luciferase virus. 48 h after inoculation, cells were harvested for RNA isolation. G3BP1 mRNA was measured by qPCR, and levels were normalized to a housekeeping gene. (B) 293T cells transfected with shRNA-3 targeting G3BP1 (50 ng, 25 ng, 12.5 ng and 6.25 ng) or shRNA control (50 ng), subsequently inoculated with VSV-g/NL4-3Luc and luciferase levels were measured 48 h later. Bars represent the average luciferase activity of triplicate measurements and the error bars represent the standard error of the mean (SEM); (C) 293T cells were transfected with shRNA targeting G3BP1(50 ng, 25 ng, 12.5 ng and 6.25 ng) or shRNA control (50 ng), together with the HIV-1 LTR Luciferase reporter construct (10 ng); luciferase levels were measured 48 h after transfection. Bars represent the average luciferase activity of triplicate measurements and the error bars represent the Standard Error of the Mean (SEM). The results from experiments show in A–C are representative of 3 independent experiments. (D and E) 293T cells were transfected with 50 ng of shRNA targeting G3BP1 or shRNA control and subsequently inoculated with VSV-g/NL4-3Luc. Proviral DNA (D) and HIV-1 Pol mRNA (E) were measured in DNA and RNA samples isolated 48 and 72 h post-inoculation, and levels were normalized to a housekeeping gene. The results from experiments show in (D and E) are representative of 2 independent experiments. (F and G) 293T cells were transfected with 100 ng of pNL4-3BaL and 100 ng of shRNA targeting G3BP1 or shRNA control. (F) Capsid protein p24 was measured in supernatant samples collected every day, by ELISA. Differences between shRAN-G3BP1 treatment and the control were analyzed by linear regression. (G) Cell lysates were collected and p24 and β-actin protein levels were measured by Western blot, in triplicate and quantified. The levels of p24 are relative to β-actin. Significant differences are indicated by asterisks (one-way ANOVA test and subsequent Bonferroni's multiple comparison test, Students *T*-test, $p < 0.05^{*}$, $p < 0.01^{**}$, $p < 0.001^{***}$).

viruses. Silencing of G3BP1 affected HIV-1 replication, but did neither affect replication of SIVmac nor MLV (Fig. 3A). Similarly, G3BP1 down-regulation only affected HIV-1 LTR-driven luciferase expression (Fig. 3B), whereas SIVmac-LTR and MLV LTR-dependent luciferase activity was not altered (Fig. 3C-D). G3BP1 down-regulation enhanced luciferase expression driven by Hepatitis B Virus S1 promoter, although the difference was not statistically significant; whereas it did neither affect expression driven by the HBV core promoter, Cytomegalovirus (CMV) promoter nor the human elongation Factor 1α (eF1α) promoter (Fig. 3E–H). These data strongly indicate that G3BP1 restricts HIV-1 replication at the level of post-integration, but does not affect replication of other retroviruses.

G3BP1 binds HIV-1 RNA

G3BP1 is a single-strand-specific endonuclease capable of binding mRNA transcripts at the 3′UTR inside stress granules (Tourriere et al., 2003, 2001) that control mRNA translation during cellular stress

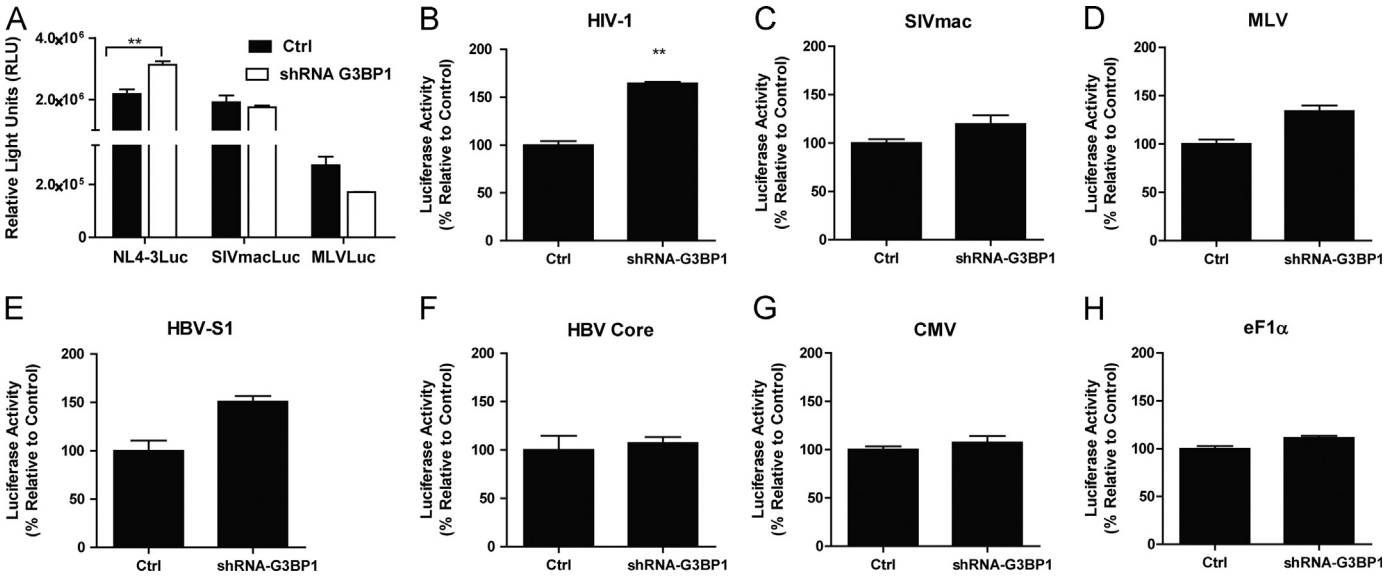

Fig. 3. G3BP1 restricts HIV-1 but does not restrict other retroviruses. (A) 293T cells were transfected with 50 ng of shRNA targeting G3BP1 or shRNA control and subsequently inoculated with single round-luciferase reporter retroviruses. Luciferase activity was measured 48 h after inoculation. The results presented are representative of two independent experiments. (B–H) 293T cells were transfected with 50 ng of shRNA targeting G3BP1 or an shRNA control and 10 ng of luciferase reporter constructs containing the different promoter regions: (B) HIV-1; (C) SIVmac; (D) MLV, (E) HBV-S1; (F) HBV Core; (G) CMV; (H) eF1α. Luciferase levels were measured 48 h after transfection. The results presented here are from two independent experiments, performed in triplicate. Bars represent the average values of two separate experiments and the error bars represent the standard error of the mean (SEM). Significant differences are indicated by asterisks (Student's *T* test; $p < 0.05^{*}$, $p < 0.01^{**}$, $p < 0.001^{***}$).

(Ortega et al., 2010; Ward et al., 2011). Therefore, we hypothesized that G3BP1 binds and sequesters HIV-1 transcripts inside cytosolic organelles, thus delaying translation and synthesis of viral proteins. To investigate this, we performed RNA-immunoprecipitation (RIP) assays in 293T cells transfected with a DNA plasmid encoding the full length HIV-1 NL4-3BaL genome. Strikingly, our results show that G3BP1 interacted with all HIV-1 RNA transcripts (unspliced, single spliced or multiple spliced) and upon silencing of G3BP1, a lesser amount of HIV-1 transcripts was immunoprecipitated (Fig. 4A). As a positive control, we were able to detect c-Myc mRNA (canonical G3BP1 mRNA target) bound to G3BP1 in all samples. We were able to confirm that there was no unspecific precipitation of cellular RNA or G3BP1 by including normal IgG antibodies as RIP negative control. Additionally, the absence other mRNAs, like β-actin or Trim5α, allowed us to rule out unspecific binding of G3BP1 to cellular RNAs. These data strongly suggest that G3BP1 binds to HIV-1 transcripts.

To determine whether G3BP1 also binds to viral transcripts in primary infected cells, we performed RIP experiments on HIV-1 infected macrophages cultured with or without IFNγ+TNFα, and measured the different HIV-1 RNA transcripts. Notably, viral RNA transcripts were bound by G3BP1 at higher levels in IFNγ+TNFα stimulated macrophages, compared to unstimulated cells (Fig. 4B).

G3BP1 is a known component of stress granules (Tourriere et al., 2003), and therefore we examined weather G3BP1 binds HIV-1 RNA inside these organelles. We analyzed expression of G3BP1 and the stress granule protein components G3BP2 (Matsuki et al., 2013), TIA-R, TIA-1 (Kedersha et al., 1999), YBX-1 (Yang and Bloch, 2007) and eIF3 (Kedersha et al., 2002) in IFNγ+TNFα-stimulated macrophages by confocal fluorescence microscopy, and compared them to macrophages containing arsenic-induced stress granules (Fig. 4C). Both G3BP1 and G3BP2 were localized in the cytoplasm of the macrophages and partially co-localized; G3BP2 was found in perinuclear, granular organelles that did not contain G3BP1. Expression of TIA-R, TIA-1, YBX-1 and eIF3 was very low and diffuse on these macrophages and did not co-localize with G3BP1 (images of separate channels for each protein can be found in the Supplementary material S1A–S1E). Notably, these stress granule proteins were found to co-localize only in stress granules induced by sodium arsenite treatment (indicated by the arrows, Fig. 4C). We also examined unstimulated macrophages upon HIV-1 infection, or in conjunction with IFNγ+TNFα stimulation, but we did not observe the formation of stress granules under these conditions either (Supplementary material S1A–S1E). These results indicate that the effect of G3BP1 on HIV-1 replication is independent of stress granule formation or other stress granule proteins.

G3BP1 restricts HIV-1 replication in primary T-cells and macrophages

$CD4^+$ T-cells are major targets for HIV-1 infection and can become latently infected by the virus. Therefore, we analyzed whether G3BP1 also restricts HIV-1 replication in primary T-cells. G3BP1 was highly expressed in resting $CD4^+$ naïve T-cells ($CD27^+CD45RO^-$) and memory T-cells ($CD27^+CD45RO^+$) obtained from healthy blood donors (Fig. 5A) and no differences in the expression levels were observed between both subpopulations. In addition, G3BP1 was also highly expressed in resting T-cells isolated from HIV-1 infected patients (participants of the Amsterdam Cohort Studies), and no differences in expression were observed between memory and naïve cells (Fig. 5B). Since productive HIV-1 infection can only be established upon T-cell activation (Stevenson et al., 1990), we measured G3BP1 mRNA in IL-2-stimulated T-cells and observed a significant decrease in G3BP1 expression upon T-cell activation (Fig. 5C).

To further investigate whether G3BP1 controls HIV-1 replication in primary target cells, we silenced G3BP1 mRNA expression in $CD4^+$ T-cells and measured replication of the VSV-g pseudotyped GFP-reporter virus (Fig. 5D–F). Strikingly, silencing of G3BP1 resulted in higher HIV-1 replication (Fig. 5D). We confirmed that silencing resulted in lower G3BP1 mRNA levels (Fig. 5E), and observed higher Pol mRNA upon silencing (Fig. 5F). We also silenced G3BP1 expression in primary macrophages and measured replication of HIV-1 VSV-g pseudotyped/Luciferase-reporter virus (Fig. 5G–I). Similarly, silencing of G3BP1 in macrophages resulted in higher HIV-1 replication, lower G3BP1 levels and higher Pol mRNA levels (Fig. 5G–I). Furthermore, we observed that macrophages produced higher amounts of infectious viral particles when G3BP1 was silenced (Fig. 5J).These results demonstrate that HIV-1 replication increases upon silencing of G3BP1 expression in

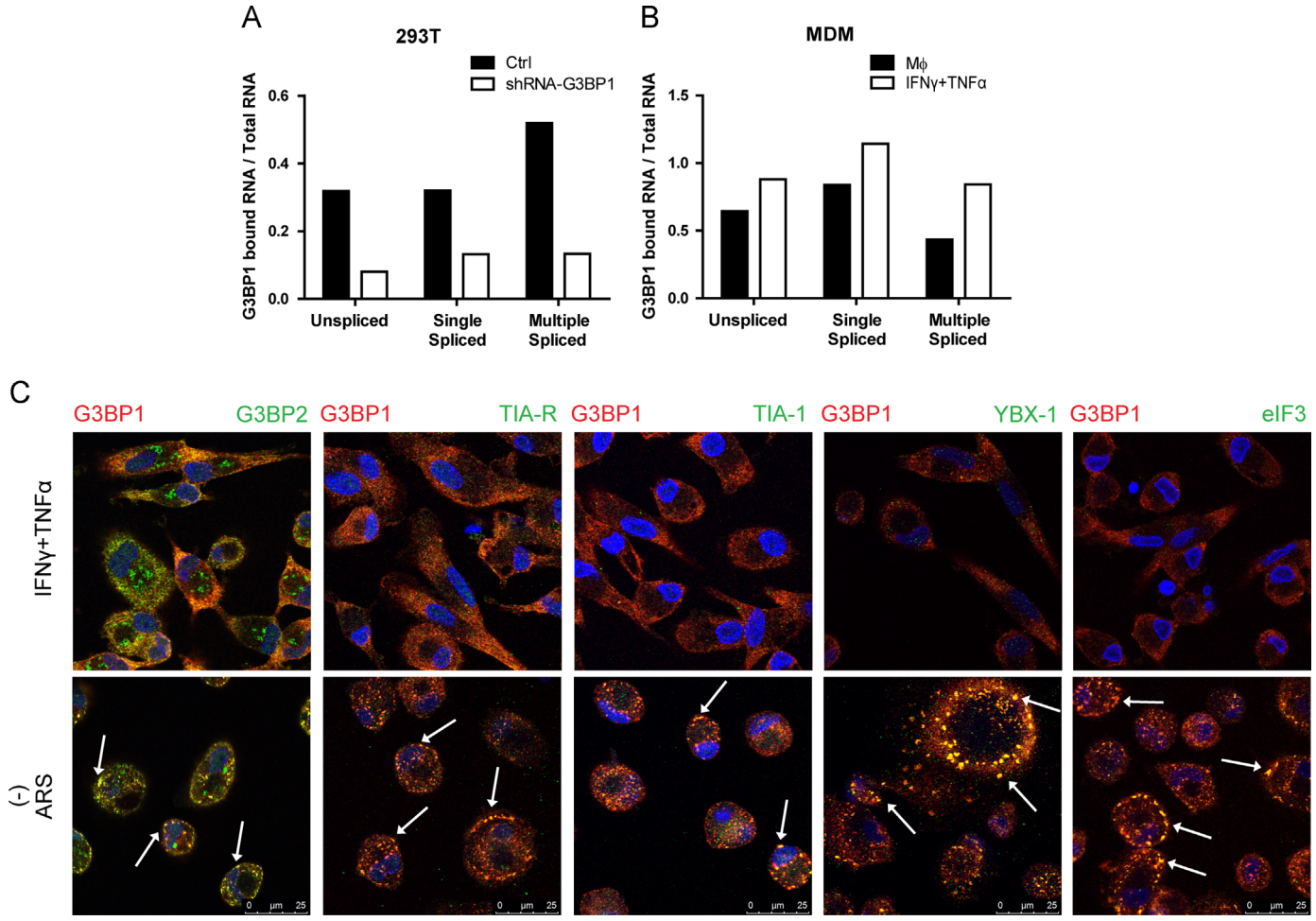

Fig. 4. G3BP1 binds to HIV-1 RNA transcripts independent of other stress granule proteins. (A) 293T cells were transfected with pNL4-3BaL and shRNA targeting G3BP1 or shRNA control, and samples were harvested for RIP 24 h post-transfection. Detection of the different HIV-1 RNA transcripts after cDNA synthesis was performed by qPCR in triplicate. Values of the ratio of transcript levels cDNA obtained after RIP compared to levels in total RNA before RIP are given. (B) Monocytes were culture with or without IFNγ+TNFα for 5 days and infected with HIV-1 NL4-3BaL, and samples were harvested for RIP 72 h post-infection. Detection of the different HIV-1 RNA transcripts after cDNA synthesis was performed by PCR and agarose-gel electrophoresis, and the intensity of the PCR product band was quantified. Transcripts levels cDNA obtained after RIP were compared to levels in total RNA before RIP. Values presented here correspond to log2 transformed ratio of the intensity of the PCR products. (C) Monocytes were culture with IFNγ+TNFα for 5 days, or treated with Sodium Arsetine (ARS). Cellular localization of G3BP1 (in red) and stress granule proteins G3BP2, TIA-R, TIA-1, YBX-1 and eIF3 (in green) was analyzed by laser scanning confocal microscopy, 24 h post-infection. Nuclei were stained with Hoechst (in blue). Merged images of all channels are shown. Images of separate channels can be found in Supplementary material S1A–S1E. White arrows point to stress granule organelles where the two stress granule proteins co-localize.

primary macrophages and T-cell, and strongly indicate that G3BP1 restricts viral replication in these cells.

Discussion

In this study, we have discovered that human G3BP1 inhibits HIV-1 replication after integration of the DNA provirus. We have shown that G3BP1 is induced by IFNγ+TNFα in macrophages and that it is highly expressed in resting T-cells. Silencing of G3BP1 in macrophages and primary T-cells increased viral replication and HIV-1-mediated gene expression, as well as viral mRNA and protein levels, and production of infectious particles. Our results demonstrate that G3BP1 binds HIV-1 full length and spliced RNA transcripts in the cytosol of infected macrophages, suggesting that G3BP1 captures viral transcripts preventing translation or for packaging into viral particles.

In our previous studies, we have shown that IFNγ+TNFα stimulation of macrophages results in inhibition of viral replication at late stages of the HIV-1 replication cycle (Cobos Jiménez et al., 2012). Therefore we used these cells to identify novel cellular factors that act at late stages of the viral replication cycle. Transcriptome analysis allowed us to select genes that are differentially expressed among activated macrophages. Among the genes that were exclusively regulated in IFNγ+TNFα stimulated macrophages, but not during differentiation of monocytes into macrophages or upon activation with other cytokines (IL-4, IL-10 and M-CSF), 19 genes have previously been indicated to possess antiviral characteristics or were involved in viral infections (Table 1). One of these genes, MX1, has recently been reported to be an IFN-α induced gene, capable of inhibiting influenza virus but not HIV-1 replication (Goujon et al., 2013). Accordingly, we identified G3BP1, which acts at a post-integration step and captures viral RNA transcripts, therefore preventing viral protein synthesis and virus particle production.

Our results strongly suggest that G3BP1 specifically inhibited for HIV-1 replication, since silencing of G3BP1 did not affect replication of other retroviruses such as SIVmac or MLV. Similarly, while G3BP1 affects HIV-1 LTR-driven gene expression, it did not alter other viral or cellular promoter activity, including SIVmac and MLV promoters. This suggests that specific motifs in the HIV-1 promoter region that are absent in the other viral promoters, allow G3BP1 to bind and capture HIV-1 transcripts in the cytosol. Our

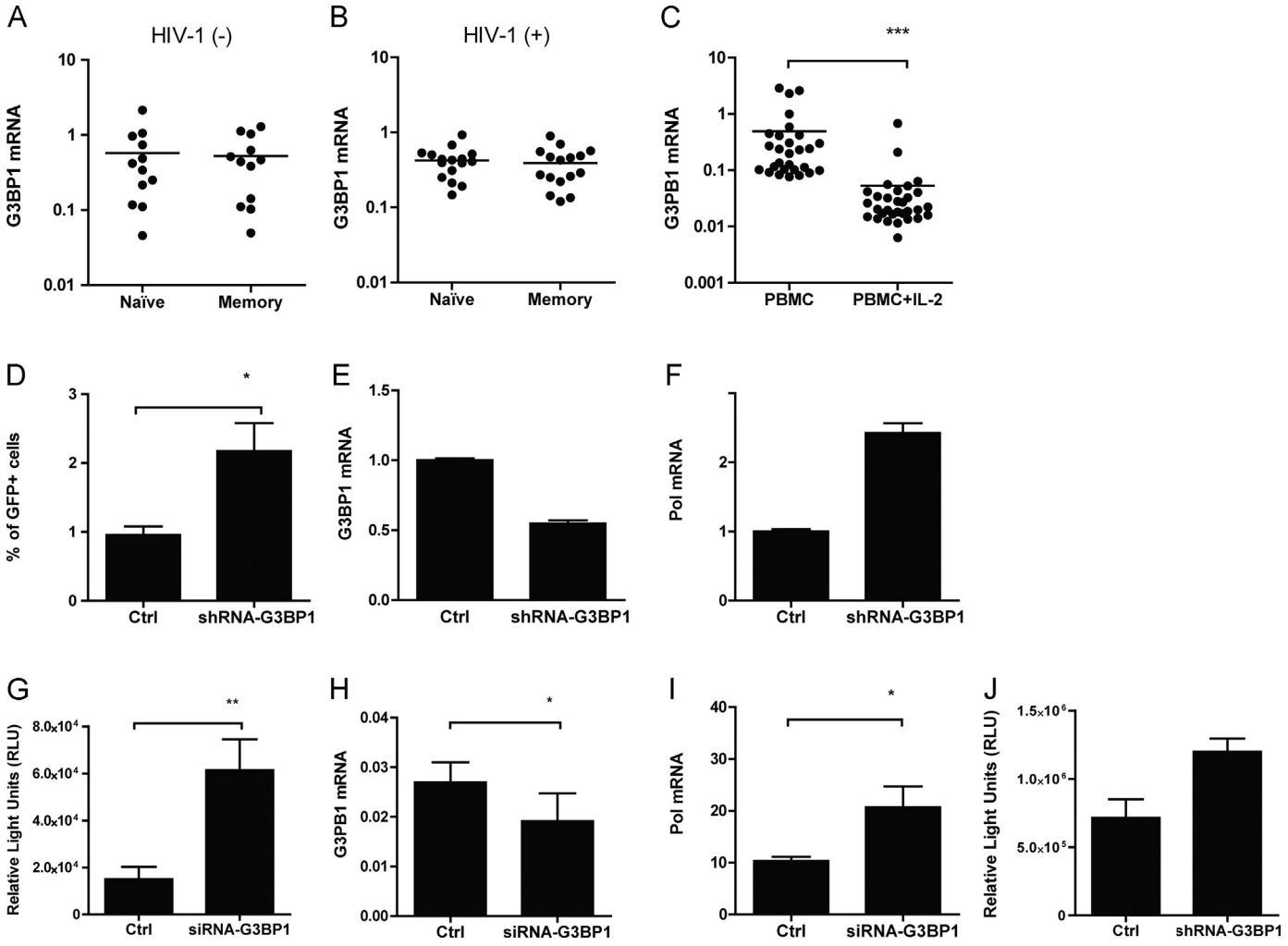

Fig. 5. Role of G3BP1 in primary T-cells and macrophages. (A) Expression of G3BP1 relative to β-actin, in memory and naïve T-cells derived from healthy blood donors or (B) HIV-1 seropositive patients, was measured by qPCR in triplicate and levels were normalized to a housekeepign gene. Each dot represents result from independent donors. (C) Expression of G3BP1 relative to a housekeeping gene, in PBMC and PBMC stimulated with IL-2. (D–F) PBMC isolated form 2 donors were inoculated with the VSV-G/YU2-GFP virus and 24 h later transduced with lentivirus expressing shRNA control or shRNA against G3BP1; (D) GFP positive cells were measured 4 days later; (E) to confirm G3BP1 downregulation, G3BP1 mRNA levels relative to a housekeeping gene were measured in cells from one donor; (F) to show the effect of G3BP1 downregulation on HIV-1 replication in $CD4^+$ T-cells, HIV-1 Pol mRNA levels relative to a housekeeping gene were measured in cells from one donor. These results are representative from 3 independent experiments. (G–I) Monocytes from 3 donors were cultured for 4 days and subsequently transfected with siRNA libraries targeting G3BP1 or non-targeting siRNAs as control, and inoculated with the VSV-G/NL4-3Luc virus at day 7; (G) luciferase levels were measured 72 h after inoculation. (H) To confirm G3BP1 downregulation, G3BP1 mRNA levels relative to a housekeeping gene were measured; (I) to show the effect of G3BP1 downregulation on HIV-1 replication in macrophages, HIV-1 Pol mRNA levels relative to a housekeeping gene were measured. These results are representative from 3 independent experiments. (J) Monocytes from 4 donors were cultured for 5 days and subsequently transduced with lentiviruses expressing shRNA control or shRNA against G3BP1. Cells were infected 48 h later with VSV-G-NL4-3BaL. Supernatant samples were collected after 24 days and assessed for infectious virus production in TZM-bl reporter cells. Significant differences are indicated by asterisks (A-C, H, I, J: Student's *T*-test, G: Mixed linear model, repeated covariance: Diagonal, D: Ratio paired *T*-Test; $p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$).

study is the first to report the restriction of G3BP1 on HIV-1 through its interaction with viral transcripts, rather than inhibiting their transcription or translation; yet the complete mechanism of action of G3BP1 still needs to be elucidated in detail.

G3BP1 is required for stress granule formation and controls mRNA translation (Tourriere et al., 2003). We demonstrate that G3BP1 is able to bind HIV-1 RNA transcripts independently of stress granule formation or other stress granules proteins. Formation of stress granules and their components are known to interact with viruses, resulting in either enabling or inhibiting viral replication (Reineke and Lloyd, 2013). It has been described that PKR (or eukaryotic translation initiation factor 2 alpha-kinase) is also required for stress granule formation upon activation by ds-RNA. The interaction between PKR, G3BP1 and RIG-I-like receptors inside stress granules is required for IFN-β production during infection with Influenza A virus lacking the NS1 viral protein (Onomoto et al., 2012). Interaction of all these components may facilitate recognition of viral RNA in the cytoplasm by RIG-I-like sensors (Onomoto et al., 2012), indicating a strong relationship between G3BP1, stress granules and innate immune responses. However, our data strongly suggest that G3BP1-mediated restriction of HIV-1 is not mediated via stress granules, since G3BP1 did not co-localize with these organelles in macrophages. In fact, a recent study (Valiente-Echeverria et al., 2014) has shown that HIV-1 is able to inhibit canonical stress granule formation, and that the interaction of G3BP1 and HIV-1 Gag mediates disassembly of stress granules. In this report, the authors indirectly show that G3BP1 is found together with viral RNA in PBMCs isolated from HIV-1 infected patient that progresses to AIDS (Valiente-Echeverria et al., 2014) but not associated with stress granules. This study lends credence to our findings that G3BP1 can interact with HIV-1 RNA outside stress granules, and also indicated that G3BP1 can also interact with other viral proteins.

Moreover, G3BP1 can also mediate innate immune responses independently of its function related to stress granules. G3BP1, together with G3BP2 and CAPRIN1 (also an RNA binding protein,

RBP) mediate mRNA translation of several Interferon Stimulated Genes (ISGs), and thus induce IFN-β production and an antiviral response against Dengue Virus (DENV), independently of stress granule formation (Bidet et al., 2014). Dengue Virus is able to bypass this mechanism by producing decoy viral RNAs (sfRNA) that bind to these RBPs, and thereby prevent mediation of the interferon response (Bidet et al., 2014). Our data strongly suggest that G3BP1 inhibits HIV-1 replication by binding HIV-1 RNA transcripts, suggesting it could also be involved in hampering the interferon response in primary HIV-1 target cells and thereby assisting HIV-1 in escaping induction of these immune responses.

HIV-1 reservoirs in primary target cells are a major hurdle in HIV-1 eradication, since they allow continuous viral replication. Our findings indicate that while HIV-1 can replicate in primary cells, replication is restricted in cells that express high levels of G3BP1 such as resting T-cells and IFNγ+TNFα-activated macrophages. Thus, it is possible that G3BP1 limits translation of HIV-1 mRNA in resting cells, but upon T-cell activation, lower G3BP1 expression may then allow efficient translation of viral transcripts, resulting in productive viral replication. Subsequent studies should definitely focus on deciphering the mechanism by which G3BP1 restricts HIV-1 and other viruses. Further research on the role of G3BP1 in primary immune cells will not only give us insight regarding HIV-1 infection and viral reservoirs but also about other infectious agents, like Influenza or Dengue virus, that also interact with G3BP1 during their replication cycle. This knowledge will also provide new opportunities to find novel targets and design new therapeutic strategies towards the elimination of the viral reservoir as part of a sterilizing cure for HIV-1 infection (Deeks et al., 2012).

Materials and methods

Isolation of monocytes and cell culture

Monocytes and peripheral blood lymphocytes (PBL) were obtained from buffy coats from healthy blood donors. Written informed consents were obtained from all donors in accordance with the ethical principles set out in the declaration of Helsinki. This study was approved by the Medical Ethics Committee of the Academic Medical Center and the Ethics Advisory Body of the Sanquin Blood Supply Foundation in Amsterdam, The Netherlands. Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats using Lymphoprep (Axis-Shield) density gradient centrifugation. Monocytes were isolated by adherence to plastic and cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% [v/v] heat-inactivated human pooled serum (HPS), penicillin (100 U/ml; Invitrogen), streptomycin (100 μg/ml) and ciproxin (5 μg/ml; Bayer) for 5 days in the presence of different cytokines: M-CSF (50 ng/ml; Sigma-Aldrich), IFN-γ (50 U/ml; Sigma-Aldrich) in combination with TNF-α (12.5 ng/ml; Peprotech), IL-4 (50 ng/ml; Peprotech), IL-10 (50 ng/ml; Peprotech), or medium alone at 37 °C in a humidified atmosphere supplemented with 5% CO_2

PBMC were also obtained from HIV-1 seropositive participants of the Amsterdam Cohort Studies. All individuals were infected with HIV-1 subtype B. The Amsterdam Cohort Studies are conducted in accordance with the ethical principles set put in the declaration of Helsinki and written consent was obtained prior to data collection. PBMC from healthy blood donors and HIV-1 positive individuals were sorted into $CD4^+$ naïve T-cells ($CD27^+CD45RO^-$) and memory T-cells ($CD27^+CD45RO^+$) by flow cytometry with the FACS Canto II (BD Biosciences). Additionally, PBMC isolated from healthy blood donors were cultured for 3 days with IMDM supplemented with 10% [v/v] heat-inactivated fetal bovine serum, penicillin, streptomycin, ciproxin and IL-2 (20 U/ml; Chirion Benelux).

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) without Hepes (Lonza), supplemented with 10% [v/v] inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37 °C in a humidified atmosphere supplemented with 10% CO_2.

Transcriptional profiles study and data mining

At day 5 the medium was removed and Monocyte Derived Macrophages (MDM) were harvested with TriPure Isolation reagent (Roche). RNA was isolated according to the manufacturer's protocol. RNA samples were cleaned with RNeasy Mini Spin columns (Qiagen RNeasy Mini Kit, Qiagen) according to the clean-up protocol from the manufacturer. The concentration was analyzed with the Nanodrop ND-1000 spectrophotometer and A260/280 values were above 2.0 for all samples. Microarrays were performed in the Illumina HumanHT-12 v4.1 Expression BeadChip kit. Background-subtracted data were normalized by Quantiles in R/Bioconductor. All datasets have been deposited in Geoprofiles: SubSeries GSE49240 (samples GSM1195728-39), part of the SuperSeries GSE35495 (Martinez et al., 2013). Genes with fold change ≥ 2 were considered differentially expressed. Statistical differences and data processing was performed in TMev v4.8.1 (Saeed et al., 2003).

qPCR

For mRNA quantification, cDNA from TriPure-isolated RNA was prepared using the M-MLV Reverse Transcriptase kit (Promega) using an oligo-dT primer, and the qPCR was performed in a Lightcycler 480II (Roche) with SYBR Green I Master or Roche Probes and Master mix (Roche). Primer sequences are available upon request. The following cycling conditions were used: denaturation for 95 °C for 10 min; amplification: 40 cycles of 95 °C for 10 s, 58 °C for 20 s and 72 °C for 30 s (Sybr green); or denaturation: 95 °C for 10 s, amplification: 55 cycles of 95 °C for 15 s, 60 °C for 60 s (Dual hydrolysis probe).

Specificity of the primer pairs was confirmed by melting curve analysis of the PCR products and relative copy numbers were calculated using the LightCycler 480 software, version 1.5. Differences in expression levels were analyzed using GraphPad Prism v5 (GraphPad software).

Viruses and expression vectors

HIV-1 NL4-3 Ba-L, single-round luciferase viruses pseudotyped with the vesicular stomatitis glycoprotein (VSV-G) (NL4-3Luc, SIVmacLuc, MLVLuc) and single round YU2-GFP virus pseudotyped with VSV-G were produced by transient transfection of HEK293T cells with pNL4-3BaL, or pCMV-VSV-G in combination with pNL4-3.Luc.R-E-, pSIVmac.Luc.R-E- (Mariani et al., 2003), pMLV-MP71Luc (Schambach et al., 2000) and helper construct pHIT60 (Soneoka et al., 1995), or pYU2, using a calcium phosphate method (He et al., 1995). Infectious virus was harvested at 48 and 72 h after transfection and filtered through a 0.22 μm filter. NL4-3 Ba-L titers were determined on PHA-stimulated PBMC as described previously (van't Wout et al., 2008). Titers of the VSV-G pseudotyped single-round luciferase and GFP viruses were determined on HEK293T cells.

pLKO.1-puro constructs expressing a shRNA against G3BP1 or shRNA control from the MISSION™ TRC-Hs 1.0 library (Root et al., 2006) were tested (1: G3BP1 TRCN0000008719, 2: G3BP1 TRCN0000008720, 3: G3BP1 TRCN0000008721, 4: G3BP1 TRCN0000008722, 5. G3BP1 TRCN0000008723 and empty vector

SHC001; Sigma-Aldrich St. Louis, MO, USA). From the 5 constructs testes, shRNA3 was the most effective in silencing G3BP1 expression, and was therefore used for subsequent experiments. Lentiviral vectors were produced by co-transfection of pLKO.1 constructs expressing shRNA-3 or the SHC001 control, and pCMV-VSV-G, pMDLgp and pRSV-Rev in HEK293T cells (Dull et al., 1998). Lentiviral vector supernatant was concentrated by centrifugation at 50,000*g* for 2 h directly after harvesting. Lentiviral vector titers were determined in HEK293T cells. Equal amounts of lentiviral vector as determined by p24 ELISA were used to inoculate target cells.

The LTR-Luciferase reporter construct pBlue3′ LTR-luc was a kind gift from Dr. R. Jeeninga and Dr. B. Berkhout, Academic Medical Center, Amsterdam, The Netherlands (Jeeninga et al., 2000). The pGL 4.51 Luc2/CMV/Neo vector (Promega) was used for evaluating luciferase expression mediated by the CMV promoter region. To generate the hEF1α-Luc construct, expressing luciferase under control of the human elongation factor 1 promoter, the luciferase gene was amplified using primers containing restriction sites for *BamHI* and *KpnI*. The amplification product was digested with these enzymes and ligated after the hEF1α promoter in a lentiviral vector digested with the same enzymes (Zufferey et al., 1998). The luciferase reporters under control of the different HBV promoters were generated as described by Du et al. (2008), with minor modifications to the primers to make them suitable to the HBV *adw* subtype. The different promoter sequences were amplified by PCR from the R9 vector, which contains a 1.2x overlength HBV DNA genome (subtype *adw*) in a pGEM 7zf+ backbone (kindly provided by Dr. Baumert) (Baumert et al., 1996). All constructs were validated by BDT sequencing.

G3BP1 silencing

Monocytes were seeded at density of 50.000 cells per well (in 96-well plates) and transfected at day 4 with 50ng of siGENOME siRNA pool libraries targeting Human G3BP1 (ThermoFisher Scientific) using DarmaFECT 4 Transfection reagent (ThermoFisher Scientific) in antibiotic-free medium containing 5% HPS, according to manufacturer´s protocol. After 48 h, the transfection medium was replaced by culture medium containing antibiotics and 10% HPS, and 24 h later cells were inoculated with 15.000 TCID50 units of VSV-g/NL4-3Luc. Luciferase activity was analyzed 72 h after inoculation as described above. Differences in luciferase levels were analyzed with a mixed linear model in SPSS 10 (IBM).

PBMCs were isolated form buffy coats and stimulated with IL-2 for 24 h. Next, $CD4^+$ cells were selected with the $CD4^+$ T-Cell Isolation Kit (Miltengy Biotec) and inoculated with the DNase treated VSV-G-YU2 virus at a MOI of 1. After 48 h, cells were transduced with lentiviruses expressing the shRNA control or shRNA-3 targeting G3BP1, at a MOI of 40. RNA samples were taken 48 h after transduction. GFP positive cells were measured 4 days after transduction by flow cytometry with the FacsCanto II (BD Biosciences) and the results were analyzed in FlowJo, version 9.4.3 (Tree Star).

Monocytes were cells were transduced with lentiviruses expressing the shRNA control or shRNA-3 targeting G3BP1, at a MOI of 40. Cells were infected with VSV-G-NL4-3BaL 48 h later, at an MOI of 0.002. Supernatant samples were collected 24 days later, and virus production was assessed on TZM-bl reporter cells.

Western blot analysis

Cells were harvested with trypsin-EDTA (PAA, Pasching Austria), collected and washed with PBS and centrifuged at 400*g* for 10 min. The cell pellet was lysed in 200 µl of RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) supplemented with Complete® EDTA free protease inhibitor (Roche). The lysate was denatured at 70 °C for 10 min in 1 × NuPAGE LDS sample buffer (Invitrogen) and 0.1 M DTT. Proteins were separated by electrophoresis on a 10% Bis-Tris gel (NuPAGE 10% Bis–Tris precast gel) together with the Odyssey Protein Weight Marker (LI-COR, Lincoln, NE, USA) using MES SDS running buffer (Invitrogen). Subsequently, proteins were transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell, Dassel, Germany; 2 h 150 V) using NuPAGE transfer buffer. Blots were stained over night at 4 °C in PBS (Gibco) supplemented with 0.01% Tween 20 (Merck) and 1% Protifar (Nutricia, Schiphol, The Netherlands) with a polyclonal goat anti-β-actin antibody (1:200; Santa Cruz) or an in-house polyclonal rabbit anti-p24 antibody (1:100). IRDye 680 conjugated donkey anti-rabbit IgG and IRDye 680 donkey anti-goat IgG (1:15.000; LI-COR, Lincoln, NE, USA) were used as secondary antibodies to visualize the proteins using the Odyssey infrared image system (LI-COR).

RNA-immuno precipitation assays (RIP)

293T cells were transfected with NL4-3BaL expression construct together with pLKO.1 construct expressing shRNA-3 targeting G3BP1 and samples were harvested 48 h after transfection. Monocytes were stimulated or not with IFNγ+TNFα for 5 days and then inoculated with HIV-1 NL4-3 Ba-L. Samples were isolated 72 h after inoculation. RNA Immunoprecipitation was performed with the EZ-Magna RIP™-RNA Binding Immunoprecipitation Kit (Millipore) together with the RIPAb$^+$™ G3BP1 – RIP Antibody and Primer set (Millipore), according to the manufacturer´s protocol. Reactions using normal mouse-IgG antibodies were used as negative controls. cDNA was synthesized form the RNA isolated after RIP, using Random Primers and the M-MLV reverse transcriptase (Promega). PCR was performed to detect HIV-1 cDNA and PCR products were visualized after 2% agarose gel electrophoresis containing Ethidium Bromide (Promega), with a UV-light transilluminator. The PCR product band intensity was quantified using the Image Studio Lite software v3.1 (LI-COR). Additionally, qPCR was also performed to detect HIV-1 cDNA using SYBRgreen MasterMix (Roche) as described above. We performed a qPCR using c-Myc primers, as a positive control for the G3BP1 canonical target, and using primers for β-actin and Trim5α as negative controls for unspecific precipitation of cellular mRNAs.

Immunofluorescence analysis

Monocytes were seeded on glass coverslips and cultured with or without IFNγ+TNFα for 5 days. Subsequently cells were inoculated with HIV-1 NL4-3 BaL for 24 h or treated with Sodium Arsenite (0.5 mM; Sigma-Aldrich) for 30 min. Cells were fixed with 1% paraformaldehyde in PBS for 30 min, and washed with PBS three times. After quenching with 10 mM glycine in PBS, cells were incubated for 20 min with methanol at −20 °C. Cells were then permeabilized with 0.1% Triton in PBS for 10 min. Coverslips were incubated with primary antibodies diluted in PBS with 5% FCS for 1 h (Mouse-anti-G3BP1, BD Transduction, rabbit-anti-G3BP2 (Assay Biotech), rabbit-anti-eIF3α, rabbit-anti-TIA-1/R (Santa Cruz), rabbit-anti-YBX1 (Bioke)). Detection of primary antibodies was done with donkey-α-mouse-Cy3 or goat-α-rabbit-Alexa488 (1:500; Jackson). Nuclei were stained with Hoechst 33342. The coverslips were mounted with Prolong Gold (Invitrogen) and analyzed using a Laser Scanning Confocal Microscope SP8-X (Leica Microsystems). Images were taken at 1024 × 1024 pixel resolution using the same settings and processed with the LAS-AFlite v2.6.0 software (Leica Microsystems).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.09.007.

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